Biochemistry

 Volume 29, Number 37 September 18, 1990

Perspectives in Biochemistry

Additivity of Mutational Effects in Proteins

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Received April 19, 1990; Revised Manuscript Received May 29, 1990

The energetics of virtually all binding functions in proteins is the culmination of a set of molecular interactions. For example, removal of a single molecular contact by a point mutation causes relatively small reductions (typically 0.5–5 kcal/mol) in the free energy of transition-state stabilization [for reviews see Fersht (1987) and Wells and Estell (1988)], protein–protein interactions (Laskowski et al., 1983, 1989; Ackers & Smith, 1985), or protein stability [for review see Matthews (1987)] compared to the overall free energy associated with these functional properties (usually 5–20 kcal/mol). Thus, it is possible to modulate protein function by mutation at many contact sites. In fact, to design large changes in function will often require mutation of more than one functional residue.

There is now a large data base for free energy changes that result when single mutants are combined. A review of these data shows that, in the majority of cases, the sum of the free energy changes derived from the single mutations is nearly equal to the free energy change measured in the multiple mutant. However, there are two major exceptions where such simple additivity breaks down. The first is where the mutated residues interact with each other, by direct contact or indirectly through electrostatic interactions or structural perturbations, so that they no longer behave independently. The second is where the mutation causes a change in mechanism or rate-limiting step of the reaction. It is important to note that the additive effects discussed here do not change the molecularity of their respective reactions. When the molecularity of the reaction changes [as in comparing the free energy of binding of one linked substrate (A–B) versus the sum of two fragments (A plus B)], large deviations from simple additivity can result from entropic effects (Jencks, 1981). Although the focus here is on enzyme activity, similar conclusions may be drawn from mutations affecting protein–protein interactions, protein–DNA recognition, or protein stability. Some practical examples and applications are discussed.

Additivity Relationships

The change in free energy of a functional property caused by a mutation at site X is typically expressed relative to that of the wild-type protein as $\Delta\Delta G_{0Y}$. Such free energy changes for two single mutants (X and Y) can be related to those of a double mutant (designated X,Y) by eq 1 (Carter et al., 1984; Ackers & Smith, 1985). The ΔG_i term (also called the coupling energy; Carter et al., 1984) should reflect the extent to which the change in energy of interaction between sites X and Y affects the functional property measured. It is possible for ΔG_i to be either positive or negative depending upon whether the interactions between the mutant side chains reduce or enhance the functional property measured. Furthermore, the ΔG_i term should not exceed the free energy of interaction between side chains at sites X and Y except in cases where these mutations cause large structural perturbations. This was first applied to evaluating the functional independence of residues mutated in tyrosyl-tRNA synthetase (Carter et al., 1984). In one case the sum of the $\Delta\Delta G$ values for single mutants was equal to that of the double mutant, indicating the sites functioned independently; in another example there was a large discrepancy, suggesting the sites were interacting.

$$\Delta\Delta G_{(X,Y)} = \Delta\Delta G_{(X)} + \Delta\Delta G_{(Y)} + \Delta G_i \quad (1)$$

Simple Additivity in Transition-State Binding Interactions

The strengths of noncovalent interactions are strongly dependent upon the nature of the two groups and the distance (r) between them. For example, the free energy of charge–charge, random charge–dipole, random dipole–dipole, van der Waals attraction, and repulsion decay as $1/r$, $1/r^3$, $1/r^4$, $1/r^6$, and $1/r^{12}$, respectively [for review see Fersht (1985)]. Thus, when the side chains at sites X and Y are remote to one another and assuming no large structural perturbations, the ΔG_i term should be negligible and eq 1 thus simplifies to

$$\Delta\Delta G_{(X,Y)} \simeq \Delta\Delta G_{(X)} + \Delta\Delta G_{(Y)} \quad (2)$$

This situation, here referred to as simple additivity, is generally observed except where side chains are close to each other or when one or both of the mutants change the rate-limiting step or reaction mechanism. These principles are well illustrated from data of additive mutational effects on transition-state stabilization energies.

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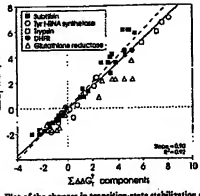

FIGURE 1: Plot of the changes in transition-state stabilization energies for the multiple mutant versus the sum for the component mutants. Data are taken from Table I and represent mutants from subtilisin (■), tyrosyl-tRNA synthetase (○), trypsin (□), DHFR (●), and glutathione reductase (▲), where mutant or wild-type side chains should not contact one another. The dashed line has a slope of 1, and the solid line is a best fit to all the data.

Changes in transition-state stabilization energy ($\Delta\Delta G^{\ddagger}_{i}$) caused by a mutation can be calculated from eq 3 (Wilkinson et al., 1983), in which R is the gas constant, T is the absolute

$$\Delta\Delta G^{\ddagger}_{i} = -RT \ln \frac{(k_{cat}/K_M)_{mutant}}{(k_{cat}/K_M)_{wild\text{-}type}} \qquad (3)$$

temperature, k_{cat} is the turnover number, and K_M is the Michaelis constant for the mutant and wild-type enzyme against a fixed substrate. $\Delta\Delta G^{\ddagger}_{i}$ represents the change in free energy to reach the transition-state complex (ES‡) from the free enzyme and substrate (E + S).

To analyze the proposition that the interaction energy term, $\Delta G^{\ddagger}_{I(X,Y)}$, is relatively small when the sites of mutation (X and Y) are remote to one another, $\Delta\Delta G^{\ddagger}_{i}$ values were collected from the literature where side-chain substitutions in the multiple mutant are beyond van der Waals contact (>4 Å distant) from each other (Table I). There are at least 25 examples distributed across five different enzymes where $\Delta\Delta G^{\ddagger}_{i}$ values can be calculated for the individual and multiple mutants assayed in at least two different ways. Among these are examples where electrostatic interactions, hydrogen bonding, and steric and hydrophobic effects have been altered separately or in combination with others. The X-ray structures of the wild-type proteins show that the wild-type side chains are not in contact. Modeling suggests the mutant side chains are beyond possible van der Waals contact unless the mutant side chains were to cause significant changes in the overall protein structure. Such large changes are rarely observed in structures of site-specific mutant proteins (Katz & Kossiakoff, 1986; Alber et al., 1987; Howell et al., 1986; Wilde et al., 1988) or even highly variant natural proteins (Chothia & Lesk, 1986).

A collective plot of the sum of the $\Delta\Delta G^{\ddagger}_{i}$ values for the component mutants versus the corresponding multiple mutant (Table I) gives a remarkably strong correlation ($R^2 = 0.92$) with a slope near unity (Figure 1). The simplest interpretation is that the interaction term, $\Delta G^{\ddagger}_{I(X,Y)}$, is small compared to the overall effects on $\Delta\Delta G^{\ddagger}_{i(X,Y)}$. It is formally possible that there are large and compensating effects between side chains X and Y that systematically lead to small net values for $\Delta G^{\ddagger}_{I(X,Y)}$.

There are some notable exceptions that weaken the correlation within the data set (Table I). In particular, combining the R204L mutation in *Escherichia coli* glutathione reductase gives a less than additive effect, especially when combined with another mutant, R198M (Scrutton et al., 1990). These basic residues are not in direct contact, but both side chains form a salt bridge with the 2′-phosphate group of NADPH. Indeed, the largest discrepancies are when these mutants are assayed with NADPH as compared to NADH. Similarly, the sum of the $\Delta\Delta G^{\ddagger}_{i}$ values for two positively charged component mutants in subtilisin (D99K and E156K) overestimates the effect of the multiple mutant when assayed with an Arg but not with a Phe substrate (Russell & Fersht, 1987). Such discrepancies are not too surprising because charge–charge interactions fall off as $1/r$ and can exhibit long-range effects in proteins [for example, see Russell and Fersht (1988)]. The physical basis for other large discrepancies not involving electrostatic substitutions is less clear but may involve unexpectedly large structural changes or changes in enzyme mechanism (see below).

These additivity tests are not particularly dominated by one of the single mutants in the sum. The average contribution (±SE) for the most dominant mutant in each sum calculated from the 69 additivity tests given in Table I is only 68% (±15%) of the total sum (theoretical is ~50%). Furthermore, the plot in Figure 1 is not analogous to graphs of correlated variables, where A is plotted versus the sum of A + B, because in Figure 1 the values on the y-axis are determined *independently* from those on the x-axis.

Complex Additivity in Transition-State Stabilization—When $\Delta G^{\ddagger}_{I(X,Y)} \neq 0$

(A) Change in Interaction Energy between Sites X and Y. Where residues X and Y are close enough to contact, it is more likely that the $\Delta G^{\ddagger}_{I(X,Y)}$ term will be significant. There are 11 examples collectively from tyrosyl-tRNA synthetase and subtilisin that fit this category (Table II).

A series of mutants in tyrosyl-tRNA synthetase at positions 48 and 51 (Carter et al., 1984; Lowe et al., 1985) show complex additivity (Table II). His48 and Thr51 in the wild-type structure are next to each other on adjacent turns of an α-helix. His48 hydrogen bonds to the ribose ring oxygen of ATP while Thr51 can make van der Waals contact with ATP. The T51P mutation increases the catalytic efficiency of the enzyme in some assays by more than −2 kcal/mol (Wilkinson et al., 1984). However, when this mutation is combined with mutations at position 48, the effects are not simply additive. An X-ray structure of the T51P mutant indicates there are no structural changes in the α-helix (Brown et al., 1987). Instead, it is suggested that the T51P mutant is improved over wild type because the wild-type enzyme contains a bound water in the vicinity of Thr51 that disfavors substrate binding. Blow and co-workers (Brown et al., 1987) argue that the change in solvent structure propagated to position 48 may account for the complex additivity. In the previous section, the double mutant (H48G,T51A) exhibited nearly simple additivity (Table I). Presumably, the smaller and less hydrophobic alanine substitution at position 51 should not introduce as large a change in solvent structure as the pyrrolidone ring of proline.

In the case of subtilisin (Table II), Gln156 is near the top of the P1 binding crevice while Gly166 is at the bottom. In the wild-type enzyme these sites do not make direct van der Waals contact, but large side chains substituted at position 166 can be modeled to contact the residue at position 156. In fact, X-ray structural analysis shows that an Asn side chain at position 166 makes a good hydrogen bond with Gln156 (Bott et al., 1987). Moreover, all of the substitutions are polar or charged, the energetics of which are expected to be the most long range. Thus, the mutant side chains alter substantially the intramolecular interactions between positions 156 and 166.

Table I: Comparison of Sums of $\Delta\Delta G_T$[a] from Component Mutants vs the Multiple Mutant Where the Mutant or Wild-Type Side Chains *Do Not* Contact One Another

assay	$\Delta\Delta G_T$[a] component mutants		sum	multiple mutant
	Tyrosyl-tRNA Synthetase			
	C35G + H48G[c]			
ATP/PP_i	+1.20	+1.04	+2.24	+2.30
ATP/tRNA	+1.05	+1.13	+2.18	+1.68
Tyr/PP_i	+1.14	+1.12	+2.26	+2.32
Tyr/tRNA	+0.32	+1.12	+1.45	+1.20
	C35G + T51P			
ATP/PP_i	+1.20	−1.91	−0.71	−1.14
ATP/tRNA	+1.05	−2.35	−1.30	−1.88
Tyr/PP_i	+1.14	−0.64	+0.50	−0.74
Tyr/tRNA	+0.32	+0.50	+0.82	+0.21
	C35G + T51C[b]			
ATP/tRNA	+1.05	−0.93	+0.12	−0.22
ATP/Tyr	+1.14	−0.91	+0.23	−0.13
	H48N + T51A[c]			
ATP/PP_i	+0.26	−0.38	−0.12	+0.04
ATP/tRNA	−0.13	−0.32	−0.45	−0.37
	T40A + H45G[d]			
Tyr/Tyr	+5.02	+3.15	+8.17	+6.95
ATP/Tyr	+5.13	+2.44	+7.57	+6.67
	Rat Trypsin			
	G216A + G226A[e]			
K	+2.75	+3.13	+5.88	+5.07
R	+2.19	+4.91	+7.10	+5.90
	Dihydrofolate Reductase ($\Delta\Delta G_{binding}$)			
	F31V + L54Q[f]			
H_2F	+1.6	+2.9	+4.5	+4.5
MTX	+2.2	+2.9	+5.1	+4.5
	Subtilisin BPN'			
	E156S + Y217L + G169A[g]			
E	−1.43	−0.87	−0.62	−2.92	−2.06
Q	−0.60	−0.36	−0.32	−1.28	−1.14
A	−0.15	−0.41	−0.27	−0.83	−0.92
K	+1.70	−0.08	−0.30	+1.32	+0.87
M	−0.86	−0.32	−0.39	−1.57	−1.41
F	−0.61	−0.29	−0.66	−1.56	−1.17
Y	−0.24	−0.12	−0.41	−0.77	−0.59
	E156S + Y217L				
E	−1.43	−0.87		−2.30	−1.67
Q	−0.60	−0.36		−0.96	−0.96
A	−0.15	−0.41		−0.56	−0.53
K	+1.70	−0.08		+1.62	+1.33
M	−0.86	−0.32		−1.18	−1.11
F	−0.61	−0.29		−0.90	−0.84
Y	−0.24	−0.12		−0.36	−0.31
	E156S, Y217L + G169A				
E	−1.67	−0.62		−2.29	−2.06
Q	−0.96	−0.32		−1.28	−1.14
A	−0.53	−0.27		−0.80	−0.92
K	+1.33	−0.30		+1.03	+0.87
M	−1.11	−0.39		−1.50	−1.41
F	−0.84	−0.66		−1.50	−1.17
Y	−0.32	−0.41		−0.73	−0.59
	D99S + E156S[h]				
R	+0.47	+0.77		+1.24	+1.52
F	0	−0.62		−0.62	−0.52

assay	$\Delta\Delta G_T$[a] component mutants		sum	multiple mutant
	Subtilisin BPN'			
	D99K + E156K			
R	+1.29	+2.12	+3.41	+2.74
F	+0.13	−0.49	−0.36	−0.42
	E156S, G166A + G169A, Y217L[i]			
F	−0.40	−1.46	−1.86	−1.76
Y	+0.94	−1.03	−0.09	+0.02
	G166A + S24C, H64A			
F	−0.40	+4.96	+4.56	+4.11
Y	+0.94	+4.40	+5.34	+5.84
	E156S, G169A, + S24C, H64A Y217L			
F	−1.46	+4.96	+3.50	+4.21
Y	−1.03	+4.40	+3.37	+3.96
	S24C, H64A, + G166A G169A, Y217L			
F	+4.21	−0.40	+3.81	+3.53
Y	+3.96	+0.94	+4.90	+6.07
	S24C, E156S, H64A, + G169A, G166A Y217L			
F	+4.11	−1.46	+2.65	+3.53
Y	+5.84	−1.03	+4.81	+6.07
	E156S, S24C, + G166A, H64A G169A, Y217L			
F	+4.96	−1.76	+3.20	+3.53
Y	+4.40	+0.02	+4.38	+6.07
	***E. coli* Glutathione Reductase**			
	A179G + R198M[j]			
NADH	−1.10	−0.62	−1.72	−1.32
NADPH	+0.08	+2.68	+2.76	+2.11
	A179G + R204L			
NADH	−1.10	+0.41	−0.69	−1.54
NADPH	+0.08	+2.42	+2.50	+0.87
	R198M + R204L			
NADH	−0.62	+0.41	−0.21	−0.51
NADPH	+2.68	+2.42	+5.10	+3.70
	A179G + R179M, R204L			
NADH	−1.10	−0.51	−1.61	−1.72
NADPH	+0.08	+3.70	+3.78	+2.22
	R198M + A179G, R204L			
NADH	−0.62	−1.54	−2.16	−1.72
NADPH	+2.68	+0.87	+3.55	+2.22
	R204L + A179G, R198M			
NADH	+0.41	−1.32	−0.91	−1.72
NADPH	+2.42	+2.11	+4.53	+2.22
	R179G + R198M + R204L			
NADH	−1.10	−0.62	+0.41	−1.31	−1.72
NADPH	+0.08	+2.68	+2.42	+5.18	+2.22

[a] Carter et al. (1984). The assays refer to measurements of ATP-dependent pyrophosphate exchange (ATP/PP_i) or tRNA charging (ATP/tRNA) under saturating conditions for tyrosine and vice versa for Tyr/PP_i exchange and Tyr/tRNA charging. [b] Lowe et al. (1985). The ATP/Tyr activation assay refers to formation of tyrosyl adenylate under saturating concentrations of tyrosine. [c] Jones et al. (1986). [d] Leatherbarrow et al. (1986). The ATP/Tyr and Tyr/Tyr activation assays refer to formation of tyrosyl adenylate under pre-steady-state conditions, and k_{cat}/K_M is calculated from k_2/K_S for tyrosine and ATP, respectively. [e] Craik et al. (1985). The substrate was D-Val-Leu-(X)-aminothiorecoumarin where the P1 residue (X) is either Lys (K) or Arg (R). [f] Adeyer et al. (1986). The ligand was either dihydrofolate (H_2F) or methotrexate (MTX). [g] Wells et al. (1987a). The substrate was succinyl-L-Ala-L-Ala-L-Pro-L-(X)-p-nitroanilide where the P1 (X) residue (Schechter & Berger, 1937) was either Glu (E), Gln (Q), Ala (A), Lys (K), Met (M), Phe (F), or Tyr (Y). [h] Russell and Fersht (1987). The substrate was benzoyl-L-Val-Gly-L-Arg-p-nitroanilide (R) or succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide (F). [i] Carter et al. (1989). The substrate was succinyl-L-Phe-L-Ala-L-His-L-(X)-p-nitroanilide where X was either Phe (F) or Tyr (Y). [j] Scrutton et al. (1990). The assay followed the reduction of oxidized glutathione by NADH or NADPH.

Table II: Comparison of Sums of $\Delta\Delta G_T^{\ddagger}$ from Component Mutants vs the Multiple Mutant Where the Mutant Side Chains Can Contact One Another

assay[a]	$\Delta\Delta G_T^{\ddagger}$ component mutants		$\Delta\Delta G_T^{\ddagger}$ sum	$\Delta\Delta G_T^{\ddagger}$ multiple mutant
	Tyrosyl-tRNA Synthetase			
	H48G + T51P[b]			
ATP/PP_i	+1.04	−1.91	−0.87	+1.07
ATP/tRNA	+1.13	−2.35	−1.22	+0.77
Tyr/PP_i	+1.12	−0.64	+0.48	+1.02
Tyr/tRNA	+1.12	+0.50	+1.63	+0.17
ATP/Tyr[c]	+0.95	−1.99	−1.04	+1.04
Tyr/ATP	+1.07	−0.38	+0.69	+0.82
	H48N + T51P			
ATP/Tyr	+0.18	−1.99	−1.81	−0.76
Tyr/Tyr	+0.36	−0.38	−0.02	−0.64
ATP/tRNA	−0.02	−2.23	−2.25	−1.07
	N48Q + T51P			
ATP/Tyr	+0.37	−0.94	−0.57	+0.86
Tyr/Tyr	+0.41	−1.00	−0.59	+0.45
ATP/tRNA	+1.26	−1.05	+0.31	+0.90
	Q48Q + T51P			
ATP/Tyr	−1.31	−1.09	−2.40	−1.22
Tyr/Tyr	−2.05	−1.65	−3.70	−2.31
ATP/tRNA	−1.87	−1.85	−3.72	−2.23
	N48Q + T51P			
ATP/Tyr	+2.26	−1.99	+0.27	+1.17
Tyr/Tyr	+3.13	−0.38	+2.75	+1.48
ATP/tRNA	+3.11	−2.23	+0.88	+1.26
	Subtilisin BPN'			
	E156Q + G166D[d]			
Q	−1.04	+1.27	+0.23	+0.75
M	−0.45	+1.83	+1.38	+0.16
K	+2.15	+0.53	+2.68	+0.26
	E156S + G166D			
Q	−0.59	+1.27	+0.68	+0.74
M	−0.85	+1.83	+0.98	+0.66
K	+1.68	+0.53	+2.22	+0.49
	E156Q + G166N			
E	−1.71	−0.11	−1.82	−0.69
Q	−1.04	+0.14	−0.90	−0.77
M	−0.45	+0.18	−0.27	−1.10
K	+2.15	+0.48	+2.73	+1.16
	E156S + G166N			
E	−1.44	−0.11	−1.55	−0.51
Q	−0.59	+0.14	−0.45	−0.85
M	−0.85	+0.18	−0.67	−0.78
K	+1.68	+0.48	+2.16	+1.26
	E156S + G166K			
E	−1.44	−3.49	−4.93	−4.49
Q	−0.59	−1.03	−1.62	−0.95
M	−0.85	−1.37	−2.22	−1.12
K	+1.68	+0.51	+2.19	+1.88
	E156Q + G166K			
E	−1.71	−3.49	−5.20	−4.49
Q	−1.04	−1.03	−2.07	−0.95
M	−0.45	−1.37	−1.82	−1.12
K	+2.15	+0.51	+2.66	+1.88

[a] See Table I for description assays. [b] Lowe et al. (1985). [c] Carter et al. (1984). [d] Wells et al. (1987b).

In these six examples there are large and systematic discrepancies between the sum of the $\Delta\Delta G_T^{\ddagger}$ values for the single mutants and those of the corresponding double mutant (Wells et al., 1987b). In almost all cases, the sum of the $\Delta\Delta G_T^{\ddagger}$ values for the single mutants is much greater than the value for the multiple mutant. Nonetheless, the $\Delta\Delta G_T^{\ddagger}$ value predicted from the sum of the single mutants does have the same sign as that for the double mutant, so that the single mutants predict qualitatively the effect on the multiple mutant.

A plot (Figure 2) of the collective data set from Table II is in contrast to that seen in Figure 1. The $\Delta\Delta G_T^{\ddagger}$ values for the multiple mutants correlate more poorly with the sum of

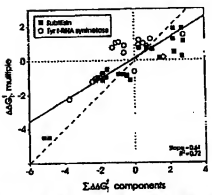

FIGURE 2: Data are taken from Table II for mutants of subtilisin (■) or tyrosyl-tRNA synthetase (○) where mutant or wild-type side chains can contact each other. The dashed line represents a theoretical line of unity slope, and the solid line represents the best fit.

the component single mutants ($R^2 = 0.72$). Moreover, the slope of the line (0.61) is much below unity. This indicates that the function of one residue is compromised by mutation of another. Of the 40 additivity examples, the average contribution of the most dominant single mutant to the sum of the $\Delta\Delta G_T^{\ddagger}$ values is 71% (±13%) of the total. Thus (as in Figure 1), both single mutants can contribute substantially to free energy changes measured in the multiple mutant. However, this data set is derived from mutations at only two different sites on two different proteins.

In summary, complex additivity can be observed when mutations at sites X and Y change the intramolecular interaction energy between sites. This can be mediated by direct steric, electrostatic, hydrogen-bonding, or hydrophobic interactions or indirectly through large structural changes in the protein, solvent shell, or electrostatic interactions. Complex additivity is most likely to occur where the sites of mutation are very close together and larger or chemically divergent side chains are introduced.

(B) Mutations at Sites X or Y Change the Enzyme Mechanism or Rate-Limiting Step. If the catalytic functions of two or more residues are interdependent, then a mutation of one residue can affect the functioning of the other(s). This form of complex additivity is well illustrated for mutations in the catalytic triad and oxyanion binding site of subtilisin (Carter & Wells, 1988, 1990). In the catalytic mechanism of subtilisin (Figure 3), the rate-limiting step is amide bond hydrolysis is transfer of the proton from Ser221 to His64 with nucleophilic attack upon the scissile carbonyl carbon. This is accompanied by electrostatic stabilization of the protonated imidazole by Asp32 and hydrogen bonding to the oxyanion by the side chain of Asn155 and the main-chain amide of Ser221. Mutational analysis shows that once the catalytic Ser221 is mutated to Ala (S221A), additional mutations in the triad or oxyanion binding site cause no further loss in catalytic efficiency (Table III).

The S221A enzyme retains a catalytic activity that is still 10^4 above the solution hydrolysis rate (Carter & Wells, 1988). It is proposed that this residual activity is derived from remaining transition-state binding contacts outside of the catalytic triad coupled with solvent attack upon the carbonyl carbon from the face opposite position 221 (Carter & Wells, 1990). This proposal is based on a model showing that there is no room for a water molecule near Ala221 once the substrate is bound. Furthermore, conversion of Asn155 to Gly enhances the activity of the S221A mutant by −1.2 kcal/mol (Table III).

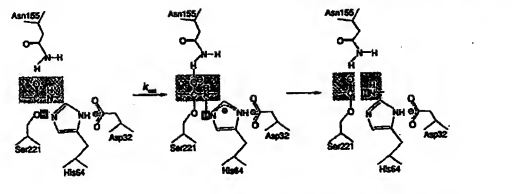

FIGURE 3: Schematic diagram of the mechanism of subtilisin showing the rate-limiting acylation step for hydrolysis of peptide bonds. Reproduced with permission from Carter and Wells (1988). Copyright 1988 Macmillan.

Table III: Comparison of Sums of $\Delta\Delta G_T$[‡] from Component Mutants vs the $\Delta\Delta G_T$[‡] for Multiple Mutants in the Catalytic Triad and Oxyanion Binding Site of Subtilisin BPN'[a]

component mutants	sum	multiple mutant
S221A + H64A[b]		
+8.93 +8.84	+17.76	+8.83
S221A + D32A		
+8.93 +6.52	+15.45	+8.86
H64A + D32A		
+8.84 +6.52	+15.36	+7.48
S221A + H64A + D32A		
+8.93 +8.84 +6.52	+24.29	+8.65
S221A + H64A, D32A		
+8.93 +7.48	+16.40	+8.65
H64A + S221A, D32A		
+8.84 +8.86	+17.70	+8.65
D32A + S221A, H64A		
+6.52 +8.83	+15.35	+8.65
S221A + N155G[c]		
+8.93 +3.08	+12.01	+7.70

[a] All enzymes were assayed with the substrate succinyl-L-Ala-L-Ala-L-Pro-L-Phe-p-nitroanilide. [b] Carter and Wells (1988). [c] Carter and Wells (1990).

This is consistent with the opposite-face solvent attack mechanism of S221A, because the oxyanion (Figure 3) would develop away from Asn155 and the N155G mutation improves solvent accessibility to the scissile carbonyl carbon.

Complex additivity is also seen for subtilisin mutated at positions 64 and 32. The double (H64A,D32A) and corresponding single mutants show a linear dependence upon hydroxide ion concentration (between pH 8 and 10) that may reflect hydroxide assistance in the deprotonation of the $O\gamma$ of Ser221 (Carter & Wells, 1988). Thus, once His64 is converted to Ala, Asp32 is a liability, presumably by electrostatic repulsion of hydroxide ion. [Note the ~1.3 kcal/mol improvement in $\Delta\Delta G_T^{\ddagger}$ for the double mutant (H64A,D32A) compared to H64A alone; Table III.]

In summary, if an enzyme mechanism relies upon cooperative interaction between two or more residues, then multiple mutations within this subset can result in large values for $\Delta G_{TD}^{\ddagger}$. In fact, if the mechanism is changed substantially, residues that were a catalytic asset can become a liability. Simple additivity can also break down when one or more of the mutations cause a change in the rate-limiting step. In an extreme case, one may have a number of mutants in an enzyme that enhance the activity, but the cumulative enhancement of activity could not go beyond the diffusion-controlled limit (Albery & Knowles, 1976).

Additive Effects on Substrate Binding

The analysis above considered changes in binding free energies between the free enzyme and substrate (E + S) to yield the bound transition-state complex (E-S*). The steady-state kinetic analysis for subtilisin and tyrosyl-tRNA synthetase is such that the K_M values approximate the enzyme–substrate dissociation constant K_s. Additivity analysis based on calculations of $\Delta\Delta G_{binding}$ (from K_M values) or $\Delta\Delta G_{act}$ (from k_{cat} values) yields qualitatively the same results (not shown) as shown in Tables I and II and Figures 1 and 2. Thus, deviations from simple additivity are not systematically found in either the energetics to form the E-S complex or those to reach E-S*.

Additive Effects on Protein–Protein Interactions

The first clear examples of additive binding effects caused by amino acid replacements in proteins were reported by Laskowski et al. (1983) and reviewed by others (Ackers & Smith, 1985; Horovitz & Rigbi, 1985). One hundred natural variants of a proteinase inhibitor, the ovomucoid third domain, have been isolated and sequenced from the eggs of different bird species (Empie & Laskowski, 1982; Laskowski et al., 1987). This is a nested set of proteins because for any one of these avian inhibitors there is a close relative containing only one or a few amino acid substitutions. Moreover, the association constants (K_a) of these inhibitors with a variety of serine proteinases vary over an enormous range (10^9-fold). Laskowski et al. (1983, 1989) have shown that the effect of a given residue replacement on K_a is about the same irrespective of the inhibitor scaffold the replacement is made in.

In addition to ovomucoid, four additivity examples have been constructed from natural variants at the subunit interface of tetrameric hemoglobin (Ackers & Smith, 1985). Three additivity examples have been analyzed for interactions of hGH with its receptor (B. C. Cunningham and J. A. Wells, unpublished results) and one example for association of synthetic variants of the RNase S peptide with RNase S protein (Mitchinson & Baldwin, 1986). The entirety of this data set is not tabulated because much on the ovomucoid inhibitors and hGH is unpublished. Nonetheless, these researchers were kind enough to provide their data formatted so it could be plotted collectively in Figure 4. These data consist of 91 additivity examples (80 in ovomucoids alone), representing 22 multiple mutants across four different proteins, and span a wide range of change in binding free energy (−10 to +7

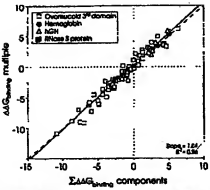

FIGURE 4: Plot showing the sum of changes in free energies of binding at protein–protein interfaces for component mutants versus the corresponding multiple mutant. Data represent interactions between ovomucoid third domain and various serine proteases (□) (R. Wynn and M. Laskowski, personal communication), regulatory interface of $\alpha_2\beta_2$ hemoglobin (●) (Ackers & Smith, 1985), hGH and its receptor (stippled Δ) (B. Cunningham and J. Wells, personal communication), and RNase S peptide and S protein (■) (Hutchinson & Baldwin, 1986). The dashed line represents a line of unity slope, and the solid line is the best fit.

kcal/mol). The plot shows a very strong linear correlation (R^2 = 0.96) with a slope near unity. Although the data for the ovomucoid were not sorted to evaluate changes at intramolecular contact sites, most are not expected to be in contact, and all of the other examples represent noncontact sites. Thus, the large data base derived from natural variants of ovomucoid third domain, as well as a smaller number of examples from several other proteins, indicates that multiple mutations at protein–protein interfaces commonly produce simple additive effects.

Additive Effects in DNA–Protein Interactions

One of the clear advantages in analyzing DNA–protein interactions is the ability to apply powerful selections that make analysis by random mutational studies feasible. Additivity in DNA–protein interactions was first demonstrated by reversion analysis of λ repressor (Nelson & Sauer, 1985). A mutation that decreased the binding affinity for the λ operator site (K4Q) was reverted by mutations at several second sites (E34K, G48S, and E83K). When these second-site revertants were introduced into wild-type λ repressor, they caused increases in affinity similar to those observed in the first-site suppressor mutant (K4Q).

Functional independence for mutations at DNA–protein contacts has been demonstrated by additive effects for mutants of CAP (catabolite gene activator protein) and its operator sequence (Ebright et al., 1987) as well as *lac* repressor and its corresponding operator sequence (Ebright, 1986). Simple additivity of mutational effects in the operator sequences for Cro repressor (Takeda et al., 1989) and λ repressor (Sarai & Takeda, 1989) has been most systematically demonstrated. Simple additivity has also been reported for multiple mutations in the *lac* repressor (Lehming et al., 1990). In fact, simple additivity is so predictable in DNA–protein interactions that the observation of complex additivity has been used to predict specific DNA–protein contacts in the *lac* repressor–operator complex (Ebright, 1986).

Additive Effects on Protein Stability

The first systematic analysis of additive effects of site-specific mutations on protein stability was reported by Shortle and Meeker (1986). Five multiple mutants in staphylococcal nuclease were constructed from a group of random single mutants that were screened initially for their ability to affect the stability of the enzyme in vivo. The component mutants do not make direct contact with each other in the multiple mutants. Generally, these variants exhibit nearly additive effects except for the double mutant V66L,G88V (Table IV). In addition to those of staphylococcal nuclease, additive effects on the $\Delta\Delta G_{unfolding}$ (assayed by reversible denaturation) have also been determined for the N-terminal domain of λ repressor (one example; Hecht et al., 1986), the α-subunit of *E. coli* Trp synthetase (one example; Hurle et al., 1986), T4 lysozyme (six examples; Wetzel et al., 1988), the gene V product of bacteriophage f1 (one example; Sandberg & Terwilliger, 1989),

Table IV: Comparison of Sums of $\Delta\Delta G_{unfolding}$ from Component Mutants vs the Multiple Mutant

assay	component mutants		sum	multiple mutant
	Staphylococcal Nuclease			
	V66L + G79S[a]			
GuHCl	−0.2	−2.6	−2.8	−3.3
urea	+0.2	−2.9	−2.7	−3.6
	V66L + G88V			
GuHCl	−0.2	−1.0	−1.2	−2.1
urea	+0.2	−0.9	−0.7	−1.4
	I18M + A69T			
GuHCl	−0.6	−2.7	−3.3	−2.8
urea	−0.7	−2.9	−3.6	−3.8
	I18M + A90S			
GuHCl	−0.6	−1.4	−2.0	−2.2
urea	−0.7	−1.4	−2.1	−2.2
	V66L + G79S + G88V			
GuHCl	−0.2	−2.6	−1.0	−3.0
urea	+0.2	−2.9	−0.9	−3.4
	N-Terminal Domain of λ Repressor			
	G46A + G48A[b]			
thermal melt	+0.7	+0.9	+1.6	+1.1
	T4 Lysozyme			
	I3C + C54V[c]			
thermal melt	+1.2	−0.7	+0.5	+0.4
	I3C + C54T			
thermal melt	+1.2	+0.3	+1.5	+1.5
	I3C + C54T + R96H			
thermal melt	+1.2	+0.3	−2.8	−1.3 −2.5
	I3C,C54T + R96H			
thermal melt	+1.5	−2.8	−1.3	−2.5
	I3C + C54T + A146T			
thermal melt	+1.2	+0.3	−1.5	0 −0.5
	I3C,C54T + A146T			
thermal melt	+1.5	−1.5	0	−0.5
	Bacteriophage f1 Gene V			
	V35I + I47V[d]			
GuHCl	−0.4	−2.4	−2.8	−2.9
	Kringle-2 of tPA			
	H64Y + R68C[e]			
thermal melt	+2.9	+0.7	+3.6	+3.4
	Turkey Ovomucoid Third Domain			
	G32A + N28S[f]			
thermal melt	+0.8	−0.5	+0.3	+0.2
	Y20H + N45-CHO			
thermal melt	−0.8	+0.3	−0.5	−0.6
	α Subunit of *E. coli* Trp Synthetase			
	Y175C + G211S[g]			
GuHCl	−0.1	+0.3	+0.2	−1.3

[a] Shortle and Meeker (1986). [b] Hecht et al. (1986). [c] Wetzel et al. (1988). [d] Sandberg and Terwilliger (1989). [e] R. Kelley, personal communication. [f] Odzewski and Laskowski (1990). N45-CHO refers to a glycosylation of Asn45. [g] Hurle et al. (1986).

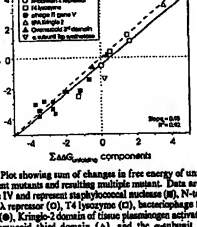

FIGURE 5: Plot showing sum of changes in free energy of unfolding of component mutants and resulting multiple mutant. Data are taken from Table IV and represent staphylococcal nuclease (■), N-terminal domain of λ repressor (○), T4 lysozyme (□), bacteriophage f1 gene V product (◆), Kringle-2 domain of tissue plasminogen activator (▲), turkey ovomucoid third domain (△), and the α-subunit of Trp synthetase (▽). The dashed line represents a theoretical line of unity slope, and the solid line represents the best fit.

natural variants of ovomucoid third domain (two examples; Otlewski & Laskowski, 1990), and the Kringle-2 domain of human tissue plasminogen activator (t-PA) (one example; R. Kelley, personal communication).

Collectively, this data set gives a high linear correlation (R^2 = 0.94) and slope near unity (Figure 5). The generally simple additive behavior is somewhat surprising given the highly cooperative nature of protein folding. There are discrepancies in some of the additivity examples besides the staphylococcal nuclease mutant (V66L,G88V). For example, the 1.5 kcal/mol discrepancy for the Y175C,Q271E double mutant in Trp synthetase (Table IV) is proposed to result from the fact that these residues are in direct contact (Hurle et al., 1986). Furthermore, proximity effects may account for the large differences between the sum of the component mutants and the multiple mutants for the α-helical double glycine mutant G46A,G48A in λ repressor (Hecht et al., 1986), and when combining R96H with the C3–C97 disulfide mutant in T4 lysozyme (Wetzel et al., 1988). In contrast, an exchange of two side chains that contact one another (V35I and I47V) in the hydrophobic core of the gene V product of f1 phage produced simple additive effects (Sandberg & Terwilliger, 1989; Table IV). It should be noted that this data base exhibiting simple additivity may be biased for single mutants that stably fold, because severely unstable proteins are more difficult to express.

By analogy to transition-state binding effects, one can certainly imagine instances where the stabilizing effects of mutations should reach a plateau. For example, denaturation at high temperatures can become controlled by a chemical step such as deamidation (Ahern et al., 1987), so that additional mutants that stabilize the folded form of the protein may be irrelevant. Another obvious example where complex additivity can be observed in protein stability is the stabilizing effect of disulfide bonds and noncovalent intramolecular contacts that require interactions between two or more residues. In these cases, the stabilizing interaction between two side chains can be broken with only one mutation.

Applications of Additivity in Rational Protein Design

A strategy of additive mutagenesis, where a series of single mutants each making a small improvement in function are combined, is one of the most powerful tools in designing functional properties in proteins. This approach has been remarkably successful in stabilizing proteins to irreversible inactivation, such as λ repressor (Hecht et al., 1986), subtilisin (Bryan et al., 1987; Cunningham & Wells, 1987; Pantoliano et al., 1989), kanamycin nucleotidyltransferase (Liao et al., 1986; Matsumura, 1986), neutral protease (Imanaka et al., 1986), and T4 lysozyme (Wetzel et al., 1988; Matsumara et al., 1989). This strategy has been applied to enhancing the catalytic efficiency of a weakly active variant of subtilisin (Carter et al., 1989), engineering the substrate specificity of subtilisin (Wells et al., 1987a,b; Russell & Fersht, 1987) and the coenzyme specificity of glutathione reductase (Scrutton et al., 1990), designing protease inhibitors with exquisite protease specificity (Laskowski et al., 1989), and recruiting human prolactin to bind to the hGH receptor (Cunningham et al., 1990). In addition, additivity principles have been used to engineer the pH profile of subtilisin (Russell & Fersht, 1987) and to design the affinity and specificity of λ repressor (Nelson & Sauer, 1985).

For this approach to work does not require that all the component mutants act in a simply additive manner but just that their effects accumulate. For example, despite the complex additivity of effects in the catalytic triad of subtilisin, there are mutagenic pathways that are energetically cumulative for installing the triad (Carter & Wells, 1988; Wells et al., 1987c). Starting with the triple mutant S221A,H64A,D32A, there is a progressive enhancement for installing Ser221 (−1.1 kcal/mol), then His64 (−1.0 kcal/mol), and finally Asp32 (−6.5 kcal/mol). Another cumulative pathway of Ser221, then Asp32, and finally His64 is possible if the Ser221,Asp32 intermediate were to use HisP2 substrates (Carter & Wells, 1987). Elaborating such cumulative pathways is important for understanding how a catalytic apparatus may have evolved and is practically useful for considering how to install such catalytic machinery into weakly active catalytic antibodies.

Conclusions

In the majority of cases, combination of mutations that affect substrate or transition-state binding, protein–protein interactions, DNA–protein recognition, or protein stability exhibits simple additivity. Simple additivity is commonly observed for distant mutations at rigid molecular interfaces such as in protein–protein and DNA–protein interactions, where the mutations are unlikely to alter grossly the structure or mode of binding.

Large deviations from simple additivity can occur when the sites of mutations strongly interact with one another (by making direct contact or indirectly through electrostatic interactions or large structural perturbations) and/or when both sites function cooperatively (as for the catalytic triad and oxyanion binding site of subtilisin). Changes at sites that can contact each other do not always lead to complex additivity; this may reflect relatively weak interactions between the two sites or indicate that the interactions are compensatory and appear to be weak.

It is important to point out the magnitude of errors in predicting the free energy effect in the multiple mutant from the component single mutants. Generally, for those cases exhibiting simple additivity (Figures 1, 4, and 5), the discrepancy in free energy between the sums of the components and multiple mutants is about ±25%. Part of this is the result of compounding errors when summing the single mutants, and the rest is presumably due to weak interaction terms. Nonetheless, this means that if the total free energy change is about 3 kcal/mol, the change in the equilibrium constant

(related by $K_{ss}/K_{eq} = 10^{-4}/RT = 155$) will often be off by a factor of 4. Thus, while the free energy effects accumulate, significant deviations will occur in predicting the final equilibrium constants when component mutants contribute a large free energy term.

Simple additivity reflects the modularity of component amino acids in protein function. This results from the fact that the perturbations in energetics and structure resulting from most mutations are highly localized. In the past six years, an additive mutagenesis strategy has been extremely effective in engineering proteins—of course, nature has been using this strategy much longer.

ACKNOWLEDGMENTS

I am grateful to Dr. Michael Laskowski and Rich Wynn for providing their data prior to publication on ovomucoid third domain and similarly to Brian Cunningham, Paul Carter, and Robert Kelley for making available their unpublished data. I am indebted for useful discussions with Drs. Michael Laskowski, Paul Carter, Jack Kirsch, and Tony Kossiakoff and many of my colleagues at Genentech and to Drs. Richard Ebright and William Jencks and those above for critical reading of the manuscript.

Registry No. RNase, 9001-99-4; tyrosyl-tRNA synthetase, 9023-45-4; trypsin, 9002-07-7; dihydrofolate reductase, 9002-03-3; subtilisin BPN', 9014-01-1; glutathione reductase, 9001-48-3; staphylococcal nuclease, 9013-53-0; lysozyme, 9001-63-2; plasminogen activator, 105913-11-9; tryptophan synthetase, 9014-52-2.

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Accelerated Publications

Role of Tyrosine M210 in the Initial Charge Separation of Reaction Centers of *Rhodobacter sphaeroides*†

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Received May 22, 1990; Revised Manuscript Received July 12, 1990

ABSTRACT: Femtosecond spectroscopy was used in combination with site-directed mutagenesis to study the influence of tyrosine M210 (YM210) on the primary electron transfer in the reaction center of *Rhodobacter sphaeroides*. The exchange of YM210 to phenylalanine caused the time constant of primary electron transfer to increase from 3.5 ± 0.4 ps to 16 ± 6 ps while the exchange to leucine increased the time constant even more to 22 ± 8 ps. The results suggest that tyrosine M210 is important for the fast rate of the primary electron transfer.

The primary photochemical event during photosynthesis of bacteriochlorophyll- (Bchl-) containing organisms is a light-induced charge separation within a transmembrane protein complex called the reaction center (RC). The crystal structures of RC's from *Rhodopseudomonas* (*Rps.*) *viridis* and *Rhodobacter* (*Rb.*) *sphaeroides* have been solved to high resolution [reviewed in Deisenhofer and Michel (1989), Chang et al. (1986), Tiede et al. (1988), and Rees et al. (1989)]. The RC from *Rb. sphaeroides* contains three protein subunits referred to as L, M, and H, according to their respective mobilities in SDS–polyacrylamide gels. Associated with the L and M subunits are the cofactors, consisting of four Bchl *a*, two bacteriopheophytin (Bph) *a*, one atom of non-heme ferrous iron, two quinones (Q_A and Q_B), and in some species one carotenoid [reviewed in Parson (1987) and Feher et al. (1989)]. The cofactors are arranged in two branches (Figure 1) with an approximate C_2 axis of symmetry. The kinetic data support a model in which the primary electron transfer proceeds after light absorption by the primary donor [a special pair of Bchl referred to as P; reviewed in Kirmaier and Holten (1987)]. The absorption of light generates the excited electronic state P^*, which has a lifetime of approximately 3 ps. An electron is transferred from P along only one branch (the so-called A-branch). It is generally accepted that after approximately 3 ps the electron arrives at the Bph on the A-side (H_A) and after 220 ps it reaches Q_A. The role of the accessory Bchl located between P and H_A (referred to as B_A) has not been definitely assigned. Recently, we have shown that at room temperature an additional kinetic ($\tau \approx 0.9$ ps) component is detectable (Holzapfel et al., 1989). The spectral properties and the kinetic constants lead to the conclusion that the corresponding intermediate is the radical pair $P^+B_A^-$ (Holzapfel et al., 1990).

Additional intriguing points concerning the process of

† Financial support was from the Deutsche Forschungsgemeinschaft, SFB 143.
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0006-2960/90/0429-8517$02.50/0